APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 9, 95–104 (1984)

Immobilized Glucose Oxidase in the Potentiometric Detection of Glucose

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Received June 13, 1983; Accepted September 15, 1983

Abstract

Previous work has shown that glucose oxidase can be immobilized on platinum to give an electrode that responds potentiometrically to glucose over the clinically useful range of about 10–250 mg glucose/100 mL. The present studies were carried out with electrochemically pretreated platinum and with gold or porous graphite substituted for the platinum support. The presence of the enzyme gave a significantly enhanced potentiometric response over that obtained with the bare support for both the pretreated platinum and the porous graphite, but not with gold. However, with platinum the potentiometric response became more negative with increasing glucose concentration. With porous graphite, the potential changed in the positive direction as the glucose concentration was increased. Hysteresis was demonstrated for the platinum–enzyme electrode. Mass transfer measurements with a rotating ring-disc electrode (RRDE) showed measurable diffusional resistances to the transport of a model electroactive compound (potassium ferrocyanide) through a matrix of immobilized enzyme attached to the disc of the RRDE. These results are part of a larger study to define the source of the potentiometric response by examining the roles of the support and the mass transfer resistances through the immobilized enzyme matrix.

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0273-2289/84/0200-0095$02.00

Index Entries: Glucose oxidase, immobilized; immobilized glucose oxidase; potentiometric determination of glucose; rotating ring-disc electrode, in glucose detection; glucose electrode; platinum–enzyme electrode, in glucose determination; enzymes, immobilized on platinum; chemically modified electrode; diabetes, detection of glucose in; gold–enzyme electrode; graphite-enzyme electrode; glucose determination, by immobilized enzyme electrode; oxidase, immobilized glucose.

Introduction

The development of a sensor for continuous in vivo monitoring of body glucose levels, without the need for removal of fluid samples, is a major goal in diabetes research. Towards this end, we have been developing a sensor that employs glucose oxidase for specificity and potentiometric measurement for detection of the products of the enzyme reaction. This work evolved from some early electrochemical studies in which glucose oxidase was immobilized on platinum and tested in glucose solutions (*1*). Other investigators have used immobilized glucose oxidase in constructing prototype in vivo glucose sensors. These authors used amperometric measurements to monitor the decrease in oxygen concentration (*2, 3*). With the amperometric-based in vivo glucose sensor, there have been problems in drift, instability, and membrane life. Clinical evaluations of these systems so far have given sporadic results. The need is still critical for alternative methods.

In earlier work we have demonstrated that potentiometric measurements of glucose concentrations can be made over the range of 10–250 mg glucose/100 mL. Normal human blood glucose levels are between 90 and 110 mg/100 mL. The following methods were used for immobilization of enzyme on the platinum: (a) entrapment in polyacrylamide gel around a platinum screen (*4*), (b) crosslinking with glutaraldehyde in the presence of bovine serum albumin (BSA) around a platinum wire (*5*), and (c) covalent coupling to platinum previously activated with an alkyl amine silane and glutaraldehyde (*6*). In most of the studies (*4, 5*) catalase was coimmobilized with glucose oxidase, and the potentiometric response was dependent on the ratio of catalase to glucose oxidase (*4*). From these studies it was concluded that the potentiometric response to glucose paralleled the concentration of hydrogen peroxide resulting from the glucose oxidase-catalyzed oxidation of glucose. However, the details of this relationship were not well understood.

In our present research we are examining four different aspects of the mechanisms of this potentiometric response. These variables are: (1) loading, activity, and reaction kinetics of the immobilized glucose oxidase; (2) mass transfer effects of the immobilization matrices; (3) surface electrochemical properties of platinum, with and without glucose oxidase; and (4) surface electrochemical properties of other electron-conducting supports with and without glucose oxidase. This paper describes the principal methodology and presents some preliminary results from these studies.

Materials and Methods

Lyophilized glucose oxidase (E.C.1.1.3.4) *ca.* 18,000 U/g solid and 20% protein/g solid from *A. niger* was obtained as Type II from Sigma (St. Louis, MO). Grade I glutaraldehyde (25% aqueous solution), β-D-glucose, and the reagents for the glucose assay (*o*-dianisidine and horseradish peroxidase) also were obtained from Sigma. Catalase from Worthington-Millipore (Freehold, NJ) was received in aqueous solution (*ca.* 0.5 mg/mL and 85,000 U/mg). The other reagents were of analytical grade. Platinum wire (0.26 mm diameter) and foils (0.051–0.025 mm thick) were obtained from Fisher Scientific (Pittsburgh PA) and Alfa Products (Danvers, MA). Porous graphite from Union Carbide (Cleveland, OH) and gold X-mat foil from Massey Bishop (Malvern, PA) were used as additional electron-conducting supports.

The glutaraldehyde–BSA–enzyme and the silane–glutaraldehyde–enzyme immobilization methods were the same as described previously (*5, 6*). When porous graphite or gold was used in place of platinum as the support, only the glutaraldehyde–BSA–enzyme method of immobilization was employed. The silane method was used to attach glucose oxidase to the disc of the RRDE.

Electrochemical preconditioning of the platinum surfaces was carried out by applying a potential of +1300 mV for *ca.* 3 min. Then the platinum was poised at a potential of −200 mV for 3 min and finally stepped to +400 mV and held for 10 min. All potentials were measured with respect to Ag/AgCl. The choice of these applied potentials is described further in the Discussion section. The electrochemical preconditioning or cyclic voltammetry measurements were carried out with a Princeton Applied Research (Princeton, NJ) Model 174A potentiostat. The platinum surfaces to be preconditioned served as the working electrode, where the current was measured. The reference and auxiliary electrodes were Ag/AgCl and a platinum screen, respectively. A medium pore-size sintered glass membrane served to isolate the auxiliary electrode from the other electrodes. The preconditioning was carried out at 25°C under anaerobic conditions, via bubbling water-saturated nitrogen through the solution. Current–potential curves were recorded on a Houston Instrument (Houston, TX) Model 2000 *X–Y* recorder.

Rotating ring–disc electrode studies were carried out with a Pine Instrument (Grove City, PA) Model ASR 2 rotator and a Model RDE3 four-electrode potentiostat. A Model DT136 rotating ring–disc electrode assembly was used. The removable platinum disc electrode, having a diameter of 0.500 cm, was separated from the platinum ring electrode by a 0.125 cm-thick epoxy spacer.

In the stationary electrode experiments, glucose oxidase or glucose oxidase/catalase were immobilized on 1.3 cm diameter pieces of platinum foil, a 1.5 cm × 2.0 cm piece of X-mat gold foil 0.23 mm thick, or a 2 × 0.5 × 0.7 cm block of porous graphite. The stationary electrode potentiometric determinations were made as described previously (*5, 6*). All potentiometric measurements were with respect to a Ag/AgCl reference electrode. When changing the glucose concentration in the stationary electrode experiments, care was taken not to move the

position of the enzyme electrode relative to the reference electrode or to expose the enzyme electrode to air. This precaution was taken because both of these factors influenced the baseline potential (i.e., plain buffer in absence of glucose). the potentiometric results are shown as the difference between the potential measured with a given glucose concentration and the baseline potential.

The main point of the RRDE studies was to determine the effects of diffusional resistances on substate mass transfer rates between the bulk solution and the platinum electrode, when different enzyme immobilization matrices were present on the platinum surface. However, this measurement could not be carried out with glucose since direct electron transfer between glucose and platinum is very slow. Therefore, potassium ferrocyanide was used in place of glucose to model the mass transfer process. In these experiments the ring electrode was held constant at +700 mV; and the potential applied to the disc electrode was scanned from −100 mV to +700 mV. The system was operated at 25°C under anaerobic conditions. The rotation of the electrode assembly produced a convective flow pattern whereby the solution was swept from the disc towards the ring. As ferrocyanide ions reached the ring, they were oxidized to ferricyanide; and the resulting ring current was measured. The relative mass transfer resistance to diffusion of ferrocyanide through the enzyme support matrix on the disc was determined by measuring the shielding coefficient. This coefficient was determined by measuring how the ring current was altered due to the oxidation of analyte as it passed the disc. The shielding coefficient is always less than unity, with the maximum theoretical value dependent on the geometry of each RRDE system.

Results

Stationary Electrode Studies

An electrochemically pretreated platinum foil was used as the support on which glucose oxidase and catalase were coimmobilized using glutaraldehyde crosslinking in the presence of BSA. A representative plot for potential response at different glucose concentrations is shown in Fig. 1 for one of these enzyme/pretreated platinum electrodes. The data are presented as the difference between the measured potential in glucose buffer and in *neat* buffer solutions. These data show that the potential of the enzyme–platinum electrode became more negative with increasing levels of glucose. The control electrode gave a much smaller potentiometric response than did the enzyme electrode. The plain platinum control electrode was pretreated in the same manner as for the enzyme electrode, but the enzyme–BSA–glutaraldehyde matrix was omitted. The difference in slopes between the enzyme electrode (−52 mV/decade of concentration) and control electrode (−17 mV/decade) is consistent with previous potentiometric data for our enzyme–platinum electrodes wherein the platinum was pretreated by other techniques (*5*, *6*). The difference in slopes also indicated that the enzyme had a marked influence on the magnitude of the potentiometric response; although a correction for the control response would be needed in a practical sensor.

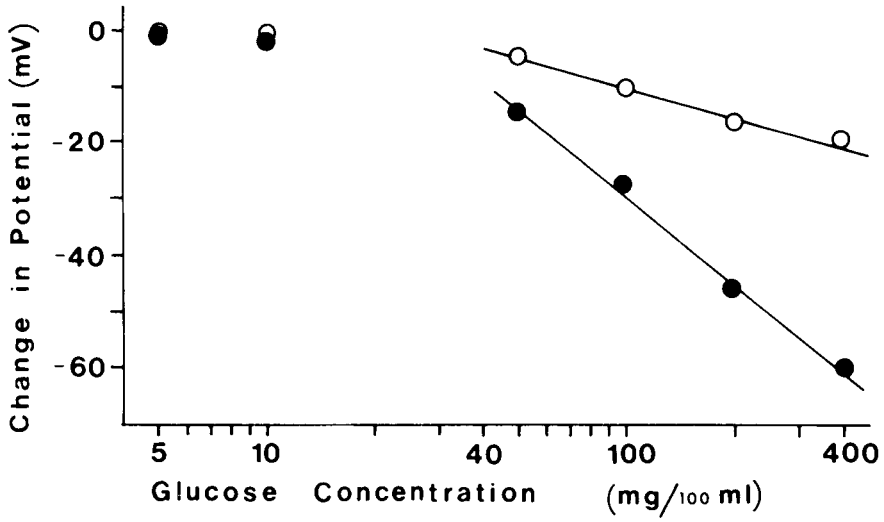

Fig. 1. Potentiometric response of platinum electrodes to glucose in 0.1*M* sodium phosphate buffer, pH 7.4, oxygen saturated, at 25°C. Enzyme electrode (●): electrochemically pretreated platinum foil with glutaraldehyde–BSA–glucose oxidase–catalase membrane. Control electrode (○): electrochemically pretreated platinum foil. Data fit by linear least squares over range of clinical interest (corr. coef.: 0.997 enzyme, 0.990 control).

Potentiometric results with the glucose oxidase/catalase system immobilized in a similar manner on a gold surface are shown in Fig. 2. The slopes in the gold electrode experiments demonstrate less difference than with platinum (−45 mV/decade with enzymes and −34 mV/decade for control). These data indicate that a gold-based enzyme electrode would be much less specific for glucose as compared to platinum.

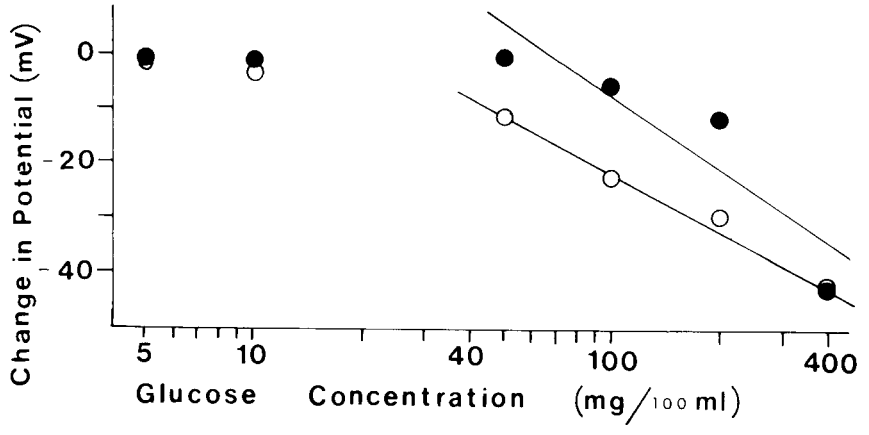

Fig. 2. Potentiometric response of gold electrodes to glucose under the same conditions as described in Fig. 1. Enzyme electrode (●): gold foil with glutaraldehyde–BSA–glucose oxidase–catalase membrane. Control electrode (○): gold foil. Data fit as in Fig. 1 (corr. coef.: 0.900 enzyme, 0.992 control).

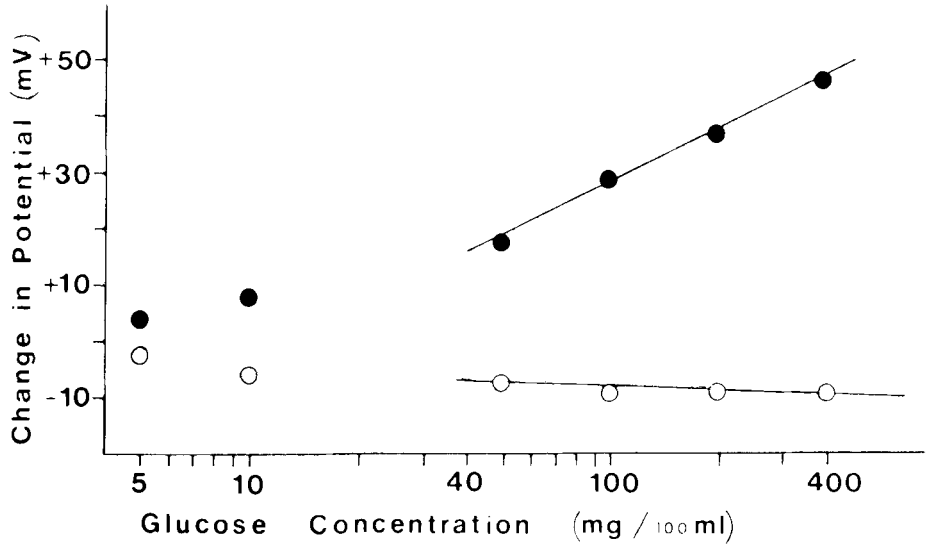

Fig. 3. Potentiometric response of porous graphite electrodes to glucose at same conditions as described in Fig. 1. Enzyme electrode (●): porous graphite with glutaraldehyde–BSA–glucose oxidase–catalase membrane. Control electrode (○): porous graphite. Data fit as in Fig. 1 (corr. coef.: 0.999 enzyme, 0.775 control).

The potentiometric response of glucose oxidase/catalase immobilized on porous graphite (Fig. 3) differed significantly from the results obtained with either the platinum or gold electrodes. The slope was positive with porous graphite and negative with platinum or gold. Thus, the mechanism(s) for generation of the potentiometric response appears to be oxidative at porous graphite and reductive at platinum or gold. The slopes differed significantly between the porous graphite/enzyme (+31 mV/decade) and porous graphite/control (−2 mV/decade), indicating good selectivity because of the enzyme.

The control electrodes also were tested for their potentiometric response in aqueous hydrogen peroxide (Fig. 4). Comparison of the representative slopes in Table 1 suggests that hydrogen peroxide may be directly involved in establishing the potentiometric response for the enzyme–porous graphite electrode, but not for the platinum or gold electrodes.

The data for Figs. 1–4 were obtained for an increasing sequence of glucose concentrations. In Fig. 5 the results are shown for an increasing sequence followed by a decreasing sequence of glucose concentrations. If no hysteresis was present, then the ascending and descending plots should superimpose on one another. These data give evidence for hysteresis in this electrode system.

Rotating Electrode Studies

These initial mass transfer studies were carried out with glucose oxidase attached to the platinum disc via a silane–glutaraldehyde linkage. The shielding coefficients for the transport of potassium ferrocyanide through the enzyme–support barrier are shown in Table 2. The silane and enzyme matrices gave a small, but measurable resistance to the transport of potassium ferrocyanide.

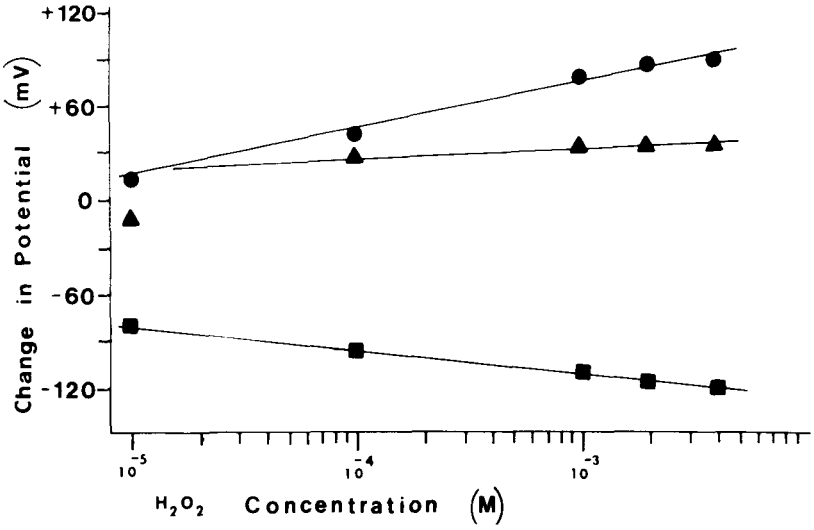

Fig. 4. Potentiometric responses of bare supports (i.e., no enzymes present) to hydrogen peroxide in 0.1*M* sodium phosphate buffer, pH 7.4, oxygen saturated, at 25°C. Platinum ■, gold ▲, porous graphite ●. Data fit by linear least squares (corr. coef.: 0.999 platinum, 0.983 gold, 0.994 porous graphite).

Discussion

Electrochemical Pretreatment of Platinum

In our earlier studies with the glutaraldehyde immobilization method (*5*), the platinum was pretreated by soaking it in 20% nitric acid; this was followed by holding it for a few seconds in a natural gas flame to a glowing red condition. However, subsequent experimental data as well as other authors suggested that the surface chemistry of the oxide film present on platinum could be an important variable in establishing the potential of our platinum–enzyme electrodes (*7* and references therein, *8*). Therefore, several different methods of electrochemical pretreatment of the platinum foils were planned. These included anodic, cathodic, and double-layer treatment. The preliminary results of the double layer pretreatment are included in this paper (Fig. 1).

The double-layer pretreatment is defined in terms of the cyclic voltammogram for platinum, as shown in Fig. 6. The anodic and cathodic currents in the vicinity

TABLE 1
Slope of Linear Portion of Potential Versus Log of Concentration

	Slope, mV/decade of concentration		
Category	Platinum	Gold	Porous graphite
Glucose and glucose oxidase/catalase electrode	−52	−45	+31
Glucose and control electrode	−17	−34	−2
H_2O_2 and control electrode	−15	+7	+31

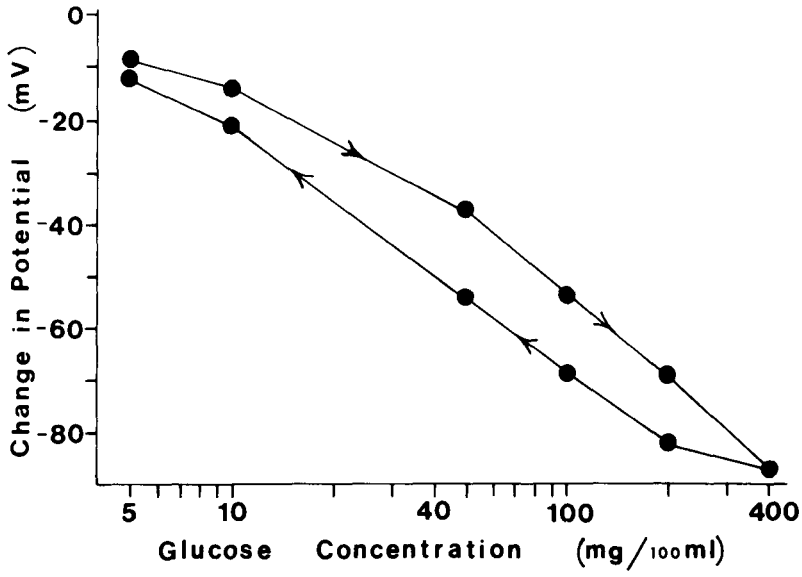

Fig. 5. Potentiometric response of platinum electrode to glucose at same conditions as described in Fig. 1. Enzyme electrode (●): platinum pretreated by holding in a natural gas flame for one minute; glutaraldehyde BSA–glucose oxidase–catalase membrane on platinum foil. Arrows indicate sequence of glucose concentations at which potentiometric readings were taken.

of +800 mV and +500 mV represent the oxidation and reduction, respectively, of the platinum surface. The currents in the region of 0 to −300 mV represent hydrogen adsorption–desorption (*7*). The double-layer pretreatment was designed to place the platinum surface in a state where neither oxidation nor reduction of the surface predominated (i.e., at about +400 mV).

Potentiometric Response of Platinum, Gold, and Porous Graphite–Enzyme Electrodes

The results in Figs. 1–3 clearly show that a potentiometric response to glucose was obtained with all three support materials, i.e., platinum, gold, and porous graphite. Only with platinum and porous graphite was the potential enhanced significantly by the presence of glucose oxidase. Therefore, two questions are pertinent

TABLE 2
Shielding Coefficients for Transport of Ferrocyanide through Matrix of Compounds Attached to Disc Surface of RRDE

Attached compounds	Shielding coefficient	Difference from theoretical,[a] %
None	0.846	0.9
Alkylamine silane	0.875	4.4
Silane–glutaraldehyde–glucose oxidase	0.870	3.8

[a]Theoretical shielding coefficient = 0.838.

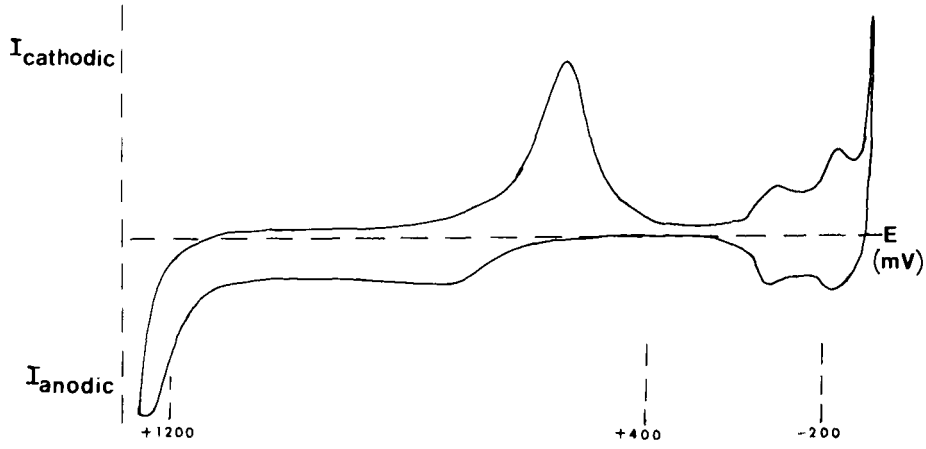

Fig. 6. Cyclic voltammogram for platinum under anerobic conditions with 0.5*M* sulfuric acid as the electrolyte. Sweep rate 2 V/min, 25°C. Potentials with references to Ag/AgCl.

to defining the practicality of this enzyme–potentiometric approach: (1) What are the sources of the potential with the plain electrodes in glucose solution? and (2) How does glucose oxidase or hydrogen peroxide lead to an augmented response in the case of platinum and porous graphite? Studies are underway with other methods of pretreatment of the platinum surface and with additional electron-conducting support materials to help answer these questions.

It has been well-documented that a thin film of platinum oxide can be found on the base metal in aqueous solutions (*8*). The stoichiometry of the platinum to oxygen ratio in this thin film, as well as the dynamics of the oxide film rearrangement and its reduction by agents such as hydrogen peroxide, is still a complex topic only partially understood (*7*). It may be that the augmented potentiometric response seen with glucose oxidase and platinum is caused by hydrogen peroxide reduction of the platinum oxide film. The concentration of hydrogen peroxide at the platinum surface presumedly depends on (1) kinetic factors influenced by the enzyme–catalyzed reaction rate, (2) mass transfer resistance for glucose and/or peroxide diffusion, and (3) catalytic rates of peroxide decomposition. The state of the platinum oxide film also may be under kinetic control influenced by the relative rates of platinum oxide formation and peroxide driven reduction. Under such kinetic control, it would not be surprising to find hysteresis in the potentiometric responses between ascending and descending glucose concentrations. ESCA studies are in progress to aid in understanding the role of the platinum surface chemistry in the potentiometric response.

Carbon surfaces are reported to have several types of functional groups, e.g., phenols, carboxylic acids, and quinones (*9*). Thus, the potentiometric response observed with the glucose oxidase–porous graphite electrode may have resulted from the oxidation of one or more carbon surface groups by hydrogen peroxide. However, it is not clear what mechanism is available for selective regeneration of reduced surface groups so as not to exhaust their surface concentration. Additional electrochemical studies are underway with carbon to elucidate the surface chemistry of the carbon/enzyme electrodes.

RRDE Mass Transport

The attachment of silane to a platinum surface is reported to provide monolayer coverage (*10*); however, it is not known whether the subsequent glutaraldehyde–enzyme addition gives a complete second monolayer or only partial coverage. In either case large resistance to mass transfer would not be expected. The results in Table 2 are consistent with this statement. Similar mass transfer studies are underway with the more complex glutaraldehyde–BSA–enzyme gel matrix. This matrix approaches complete coverage of the metal surface; and the larger resistances to mass transfer would be expected.

Acknowledgments

This work was supported by grant RO1-AM26370 from the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases of NIH. The authors appreciate the skilled technical efforts of Leslie A. Cantin in carrying out much of the experimental work.

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